# The potential role of long non-coding RNAs in key steps of *Leishmania (Viannia) braziliensis* life cycle

Caroline Ricce Espada[1], José Carlos Quilles Júnior[1], Rubens Miserani Magalhães[1], Tânia Paula Aquino Defina[1], Angela Kaysel Cruz[1].

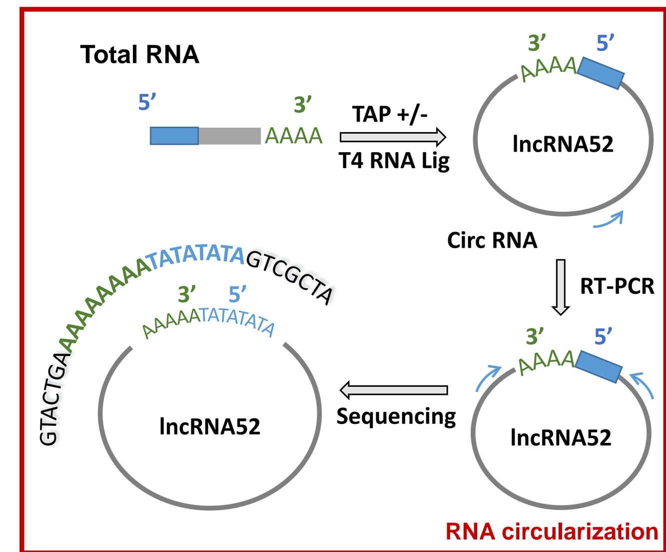
[1]Departamento de Biologia Celular e Molecular, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo FMRP-USP, Ribeirão Preto, São Paulo, Brasil.

## Introduction

*Leishmania (Viannia) braziliensis* is an important causative agent of cutaneous and mucocutaneous leishmaniasis in Americas. During its life-cycle, *Leishmania* alternates between two hosts, facing dramatic changes in environmental conditions, requiring a fast and dynamic gene expression modulation to survive. *Leishmania* lack canonical promoters for its coding genes and thus, regulation of gene expression is mostly a result of copy number variation or post-transcriptional processes. Non-protein coding RNAs (ncRNAs) were described in different organisms, including trypanosomatids, as regulatory elements acting in different processes like transcription, RNA stability and transport to the cytoplasm, methylation, acetylation, translation an others. In *L. braziliensis*, an effort to understand differential gene expression during this parasite life cycle led to the discovery of 11,372 putative ncRNAs of which at least 295 were differentially expressed in all three morphologies (procyclic promastigote, metacyclic promastigotes and axenic amastigotes). In this context, we are now investigating the role of these potential non-coding RNAs in the biology of *L. braziliensis* aiming to understand the regulation of *Leishmania* gene expression.

## Methods

Ten differentially expressed and intergenic long-ncRNAs (>200bp) were selected in a initial screening. Differential expression of the lncRNA was confirmed by real-time PCR. A **protocol of RNA circularization** was used to determine the lncRNA size. In this protocol, TAP-treated total RNA is circularized and reversed-transcribed using primers specific to the target. The resulting cDNA is then PCR-amplified using primers directed to the 5' and 3' ends of the transcript and submitted to sequencing. CRISPR/Cas9-mediated genome editing was used to delete these elements from *L. braziliensis* genome. Knocked out clones homozygous for the deletion were submitted to phenotypical tests evaluating *in vitro* growth, survival to nutritional (PBS) and oxidative stress ($H_2O_2$), metacyclogenesis rates and infectivity to THP-1 cells.




## Results

The lncRNAs were selected based on their differential expression between *Leishmania* morphologies (FC tab) and on their conservation among other *Leishmania* species (*spp*) or its exclusivity in *L. braziliensis* (*Lbr*). We successfully obtained complete knockouts for 5 out of 10 lncRNAs in *L. braziliensis* M2903 Cas9/T7 line (KO tab). The lack of 3 out of 5 elements led to alterations in *L. braziliensis* fitness. Differential expression was confirmed for those lncRNAs presenting alterations in phenotype upon deletion (with exception of lncRNA52 which is ongoing). Size, presence of a poly-A tail at 3' UTR and of a miniexon at 5' UTR were characterized by RNA circularization for lncRNAs with phenotypes. The results for each target are:

- **LncRNA52** is 3.45-fold more expressed in axenic amastigotes and its size varies from 896 to 838nt. This element presents a poly-A tail varying from 25 to 41 adenines and also a miniexon sequence was detected in 3/10 transcripts. The deletion of **lncRNA52** caused a significant reduction in metacyclogenesis rates in *L. braziliensis* M2903
- **LncRNA66** is 2.39-fold more expressed in procyclic promastigotes and its size varies from 441 to 450nt. This elements presents a poly-A tail of 12nt but no miniexon was detected. The deletion of **lncRNA66** led to a reduced growth rate and an increased doubling time in procyclic promastigotes of *L. braziliensis* M2903
- **LncRNA31** is 4.27-fold more expressed in metacyclic promastigotes and its size varies from 254 to 486nt. Neither poly-A tail nor miniexon were detected in this element. The deletion of **lncRNA31** led to a significant increased doubling time of axenic amastigotes of *L. braziliensis* M2903

| | Length | spp/Lbr | Stage | FC | KO |
|---|---|---|---|---|---|
| 26_lncRNA45 | 408 | *spp* | AMA | 1.53 | Ongoing |
| 33_lncRNA296 | 233 | *spp* | META | 1.82 | Complete |
| 01_lncRNA36 | 290 | *Lbr* | AMA | 2.03 | Ongoing |
| 22_lncRNA71 | 582 | *spp* | META | 2.33 | Complete |
| 31_lncRNA232 | 1306 | *spp* | AMA | 2.05 | Ongoing |
| 26_lncRNA66 | 323 | *spp* | PRO | 2.39 | Complete |
| 23_lncRNA170 | 1135 | *spp* | AMA | 3.53 | Ongoing |
| 11_lncRNA31 | 251 | *Lbr* | META | 4.27 | Complete |
| 01_lncRNA52 | 894 | *Lbr* | AMA | 3.45 | Complete |
| 13_lncRNA133 | 757 | *Lbr* | AMA | 2.34 | Ongoing |

Ongoing / Complete

Mammals

Female Sandfly

lncRNA66 - Promastigote Growth

lncRNA52 - Metacyclogenesis

AXA META PRO

Dark blue: without miniexon
Light blue: miniexon detected

Metacyclogenesis — % of Metacyclogenesis; WT, Δ01_lncRNA52

Unpaired T-test *p<0.05

lncRNA31 - Axenic amastigotes duplication

RT-qPCR lncRNA66 — Fold-change (xPRO); 7SL, G6PD; Pro, Meta, Ama

Pink: Predicted secondary structure

Promastigotes/mL; Days; WT, 26_lncRNA66

Promastigotes Doubling Time (h); WT, 26_lncRNA66

** t-Test p<0.004

Real-time lncRNA31 — Fold-change (xPRO); 7SL, G6PD; Pro, Meta, Ama

Promastigotes/mL; Days; WT, 11_lncRNA31

Amastigotes Doubling Time (h); WT, 11_lncRNA31

**** t-Test p<0.0001

## Conclusions

- LncRNAs 52, 66 and 31 are differentially expressed between morphologies of *L. braziliensis* M2903.
- The deletion of these lncRNAs compromised parasite fitness suggesting that these elements have a regulatory function in *L. braziliensis* M2903.
- Circularization assay suggests the these lncRNAs indeed exist as transcripts and may differ in processing (polyadenylation and capping).
- *In vitro* pull-down assays are ongoing to identify proteins interacting with these elements.

UK:Brazil Joint Centre Partnership in Leishmaniasis · FAPESP · CNPq · CAPES